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Structural and functional effects of myosin binding protein-C phosphorylation in heart muscle are not mimicked by serine-to-aspartate substitutions

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**Running Title:** *Serine-to-aspartate substitutions in cMyBP-C*

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## ABSTRACT

Myosin-binding protein-C (cMyBP-C) is a key regulator of contractility in heart muscle, and its regulatory function is controlled in turn by phosphorylation of multiple serines in its m-domain. The structural and functional effects of m-domain phosphorylation have often been inferred from those of the corresponding serine-to-aspartate (S–D) substitutions, in both *in vivo* and *in vitro* studies. Here, using a combination of *in vitro* binding assays and *in situ* structural and functional assays in ventricular trabeculae of rat heart and the expressed C1mC2 region of cMyBP-C, containing the m-domain flanked by domains C1 and C2, we tested whether these substitutions do in fact mimic the effects of phosphorylation. *In situ* changes in thin and thick filament structure were determined from changes in polarized fluorescence from bifunctional probes attached to troponin C or myosin regulatory light chain, respectively. We show that both the action of exogenous C1mC2 to activate contraction in the absence of calcium and the accompanying change in thin filament structure are abolished by tris-phosphorylation of the m-domain, but unaffected by the corresponding S–D substitutions. The latter produced an intermediate change in thick filament structure. Both tris-phosphorylation and S–D substitutions abolished the interaction between C1mC2 and myosin sub-fragment 2 (myosin S2) *in vitro*, but

yielded different effects on thin filament binding. These results suggest that some previous inferences from the effects of S–D substitutions in cMyBP-C should be reconsidered and that the distinct effects of tris-phosphorylation and S–D substitutions on cMyBP-C may provide a useful basis for future studies.

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Phosphorylation of cardiac myosin binding protein-C (cMyBP-C) is an important determinant of cardiac muscle function, and ablation of cMyBP-C phosphorylation has frequently been associated with heart failure, further underlining its functional significance (1). cMyBP-C is phosphorylated *in vivo* (2) and is a substrate for multiple protein kinases that target conserved serine residues in the m-domain or m-motif that links the C1 and C2 Ig domains (Fig. 1A) (3). Phosphorylation of these residues controls the interaction of the N-terminal region of cMyBP-C with both the myosin-containing thick and the actin-containing thin filaments (4). Interactions of cMyBP-C's N-terminal domains with myosin are thought to have mainly inhibitory effects on contractility by stabilizing the thick filament OFF state, characterized by myosin head domains bound to the filament core in quasi-helical tracks (5,6). In contrast, cMyBP-C-actin interactions have been frequently associated with an activating effect on contractility and thin

filament structure (5,7). Phosphorylation of cMyBP-C has been shown to abolish these regulatory interactions and therefore modulate cardiac contractility via changes in the thick and thin filament regulatory state.

Serine to aspartate (S-D) substitutions of serine residues within the m-motif have often been used as a proxy for phosphorylation, *in vivo* in transgenic animal models (8,9), *in situ* in isolated cardiac muscle cells (10), and *in vitro* in isolated proteins (11,12). The assumption that S-D substitutions reproduce the effects of phosphorylation in cMyBP-C has generally not been critically examined. *In vivo* experiments on transgenic animal lines do not allow direct comparison of the effects of fully phosphorylated and S-D substituted cMyBP-C on cardiac muscle function. In contrast, *in vitro* experiments using isolated proteins allow some functional mechanisms to be characterized at the molecular level, but do not reproduce regulatory and contractile mechanisms that depend on the native lattice of thick and thin filaments.

In this study, we used bifunctional fluorescent probes on myosin regulatory light chain (RLC) or troponin C (TnC) to monitor structural changes in the thick and thin filaments respectively in rat heart muscle cells. We induced activation of the muscle cells using exogenous rat cardiac C1mC2 fragments that were native, tris-phosphorylated or carrying three S-D substitutions. We combined these structural measurements with measurements of active isometric force, and with biochemical characterization of the interaction between C1mC2 and myosin S2, and C1mC2 and native thin filaments (NTF) *in vitro*, to determine whether S-D substitutions reproduce the structural and functional effects of phosphorylation.

## Results

### ***Phospho-mimetic substitutions in C1mC2 do not abolish thin filament activation***

N-terminal fragments of rat cardiac myosin binding protein-C containing the Ig-domains C1 and C2, and the intervening m-domain (C1mC2) are sufficient to activate contraction of demembrated rat ventricular trabeculae in the absence of  $\text{Ca}^{2+}$ , and tris-phosphorylation of C1mC2 by PKA (Protein Kinase A) abolishes this effect (5). This assay therefore enables a test of the ability of serine-to-aspartate (S-D) substitutions to act as phospho-mimetics for this mode of action of cMyBP-C. Trabeculae were incubated in relaxing solution (pCa 9) containing

40  $\mu\text{mol/L}$  unphosphorylated C1mC2, PKA tris-phosphorylated C1mC2 (C1mC2-3P; Fig. S1) or C1mC2 with three phosphorylatable serines mutated to aspartate (C1mC2-3SD) (Fig. 1). Incubation in C1mC2 produced an isometric force of  $57 \pm 5\%$  (mean  $\pm$  SEM,  $n=13$ ) of that during full  $\text{Ca}^{2+}$  activation (Fig. 1B), whereas C1mC2-3P produced no activation, as described previously (5). Strikingly, C1mC2-3SD activated contraction to the same extent as C1mC2 ( $57 \pm 5\%$ , mean  $\pm$  SEM,  $n=13$ ). In complete contrast to phospho-serines, the S-D substitutions have no effect on the activating effect of C1mC2.

We used bifunctional (sulfo)rhodamine probes attached to either the E-helix of cardiac troponin C (BR-cTnC-E) or crosslinking helices B and C of cardiac myosin regulatory light chain (BSR-cRLC-BC) (Fig. S2) to monitor structural changes in the thin and thick filaments, respectively, in this assay. The cTnC E-helix is part of the IT-arm of troponin and its orientation is coupled to the position of tropomyosin on the surface of the thin filament. The order parameter  $\langle P_2 \rangle$  describing the orientation of BR-cTnC-E decreases on calcium activation, suggesting a more perpendicular orientation of the E-helix (Fig. 1C). Incubation of BR-cTnC-E exchanged trabeculae in relaxing solution (pCa 9) containing 40  $\mu\text{mol/L}$  C1mC2 induced a change in thin filament structure reported by the TnC E-helix probe that is significantly larger than that during full calcium activation, as reported previously (5). In contrast with unphosphorylated C1mC2, C1mC2-3P had no effect on thin filament structure. However C1mC2-3SD had the same effect as C1mC2 (Fig. 1C). Thus, in full agreement with the functional effects described above, the S-D substitutions do not reproduce the effect of tris-phosphorylation of C1mC2 on thin filament structure.

The RLC BC-helix probe is mainly sensitive to the regulatory state of the thick filament (13), and  $\langle P_2 \rangle$  for this probe increases on calcium activation (Fig. 1D). Incubation of BSR-cRLC-BC exchanged trabeculae in 40  $\mu\text{mol/L}$  C1mC2 induced a change in BSR-cRLC-BC probe orientation of  $\sim 70\%$  of that for calcium activation, whereas C1mC2-3P produced no significant change in probe orientation. C1mC2-3SD had an intermediate effect.

The above results show that the S-D substitutions in C1mC2 do not mimic the effects of tris-phosphorylation for the active force and accompanying effects on thin and thick filament

structure induced by C1mC2 in the absence of calcium. For active force and thin filament structure, the action of C1mC2-3SD was indistinguishable from that of the native fragment. For thick filament structure, the effect was intermediate, suggesting the possibility that S-D substitutions in the m-domain might mimic phosphorylation of these residues with respect to the direct action of C1mC2 on thick but not thin filament structure, in the light of previous evidence for coupling between the regulatory states of the thin and thick filaments (5,14).

### ***Phospho-mimetic substitutions in C1mC2 alter thin filament interaction and abolish binding to myosin S2***

We used high-velocity co-sedimentation to determine the effects of phosphorylation and S-D substitutions in C1mC2 on binding to isolated native thin filaments (NTF). The unphosphorylated C1mC2 binds NTFs in a saturable manner with a  $K_d$  of  $23 \pm 7 \mu\text{mol/L}$  (mean  $\pm$  SEM,  $n=7$ ) and  $B_{\text{max}}$  of  $0.81 \pm 0.10 \text{ mol/mol}$ , suggesting roughly stoichiometric binding of C1mC2 to actin (Fig. 2A). PKA tris-phosphorylation of C1mC2 increased  $K_d$  to  $57 \pm 5 \mu\text{mol/L}$  (mean  $\pm$  SEM,  $n=8$ ) and reduced  $B_{\text{max}}$  to  $0.67 \pm 0.06 \text{ mol/mol}$ , indicating reduced binding to NTFs *in vitro*, in agreement with previously published results (15). C1mC2-3SD (Fig. 2A, purple) binds NTFs with a  $K_d$  of  $16 \pm 5 \mu\text{mol/L}$ , (mean  $\pm$  SEM,  $n=4$ ), similar to that of unphosphorylated C1mC2, but with a significantly lower  $B_{\text{max}}$ ,  $0.51 \pm 0.06 \text{ mol/mol}$ .

Next we tested whether S-D substitutions mimic the effect of phosphorylation of m-domain serines on the binding of C1mC2 to myosin S2 $\Delta$ , the first 126 amino acids of myosin S2 (16) by Microscale Thermophoresis (MST). Binding of C1mC2 to myosin S2 $\Delta$  is strongly temperature-dependent. At 21°C  $K_d$  was  $\sim 100 \mu\text{mol/L}$ , but at 30°C it was reduced to  $\sim 20 \mu\text{mol/L}$ , in agreement with previous results (16) (Fig. S3; Tab. 1). Moreover, the binding curve is biphasic, indicating an additional low affinity binding interaction with an estimated  $K_d$  of  $\sim 400 \mu\text{mol/L}$  (arrow head in Fig. S3).

As a step towards localising the binding interactions between cMyBP-C and myosin S2 $\Delta$ , we measured the binding affinity for myosin S2 $\Delta$  to the isolated m-motif, and to a construct containing Ig domains C1 and C2 connected by a flexible linker (C1-C2), i.e. with the m-motif deleted (Fig. 2B; Tab. 1). The m-motif alone

binds myosin S2 $\Delta$  with a  $K_d$  of  $\sim 40 \mu\text{mol/L}$ , indicating that it is the main interaction site between C1mC2 and myosin S2 $\Delta$ . In contrast C1-C2 binds myosin S2 $\Delta$  with a  $K_d$  of  $\sim 500 \mu\text{mol/L}$ , suggesting that the flanking C1 and C2 domains are responsible for the low-affinity binding sites in C1mC2 and only have a small contribution to C1mC2 binding to myosin S2 $\Delta$ .

Increasing ionic strength greatly reduced the affinity of C1mC2 for myosin S2 $\Delta$  (Fig. 2C; Tab. 1), suggesting that the interaction between the m-motif and myosin S2 $\Delta$  is mainly ionic. Consistent with that interpretation, binding of the isolated m-motif to myosin S2 $\Delta$  was largely abolished at the higher ionic strength.

Both PKA tris-phosphorylation and replacement of the three phosphorylatable serines by aspartate abolished the high affinity interaction between C1mC2 and myosin S2 $\Delta$  (Figs. 2D and S4; Tab. 1), as expected for the largely ionic nature of the high affinity binding site in the m-motif.

### **Discussion**

The results presented above show that serine-to-aspartate substitution of the three phosphorylation sites in the m-motif of cMyBP-C fully mimic the effects of phosphorylation of those sites with respect to binding to myosin S2 $\Delta$  but fail to mimic its effects on the interaction between cMyBP-C and the thin filament. The ionic strength dependence of the binding between cMyBP-C and myosin S2 suggests that it is mainly driven by ionic interactions between the m-motif and myosin S2 $\Delta$ , consistent with the fact that it can be abolished by the introduction of additional negative charges, by either phosphorylation or S-D substitutions. In contrast, regulatory interactions of cMyBP-C with NTFs have been localized to both domain C1 and the m-motif (17). Phosphorylation of the m-motif serines appears to control these regulatory interactions by altering C1mC2 structure (18), in a way that is not efficiently mimicked by S-D substitutions. Although S-D substitutions reduce the binding capacity of C1mC2 to NTFs to a similar extent to that observed for the effects of PKA phosphorylation on the native protein, they do not affect  $K_d$  and thin filament activation, suggesting that phosphorylation might affect thin filament activation via a structural change communicated between the m-motif and domain C1 (15).

These results in combination with previous work therefore suggest that: unphosphorylated native

cMyBP-C binds to both the thin and thick filaments (Fig. 3A), stabilizing their ON and OFF states respectively; cMyBP-C phosphorylation abolishes both regulatory interactions, so that myosin heads are released from the thick filament backbone but the thin filament is OFF (Fig. 3B), and substitution of the phosphorylatable serines by aspartates releases the myosin heads from the thick filament backbone without affecting the regulatory interaction with the thin filament (Fig 3C), therefore stabilizing the ON state of both thin and thick filaments. It follows that it could be quite misleading to interpret the effects of S-D substitutions in cMyBP-C as reproducing the structural or functional effects of tris-phosphorylation of cMyBP-C *in vitro* or *in vivo*. On the other hand the differential effects of S-D substitutions and tris-phosphorylation on the thin- and thick-filament interactions of cMyBP-C may provide a useful approach to dissect the effects of those interactions in future studies.

## Experimental procedures

### *Preparation of bifunctional rhodamine labelled cRLC and cTnC, and exchange into demembranated rat ventricular trabeculae*

Wistar rats (male, 200-250 g) were sacrificed by cervical dislocation (Schedule 1 procedure in accordance with UK Animal Scientific Procedure Act, 1986) and demembranated right ventricular trabeculae were prepared as described previously (19). All procedures were carried out in accordance with the guidelines of the Animal Welfare and Ethical Review Body (AWERB, King's College London). All animals were treated in accordance with the guidelines approved by the UK Animal Scientific procedures ACT (1986) and European Union Directive 2010/63/EU.

Bifunctional sulforhodamine (BSR) labelled RLCs and bifunctional rhodamine (BR) labelled TnCs were prepared as previously described (5,19). BSR-cRLCs were exchanged into demembranated trabeculae by extraction in CDTA-rigor solution (composition in mmol/L: 5 CDTA, 50 KCl, 40 Tris-HCl, 0.1% (v/v) Triton X-100, pH 8.4) for 30 min followed by reconstitution with 40  $\mu$ mol/L BSR-cRLC in relaxing solution for 1 h at 22°C, replacing ~50% of the endogenous RLC (5). BR-cTnCs were exchanged by overnight soak on ice of demembranated trabeculae in relaxing solution containing 0.5 mg/ml BR-cTnCs, replacing ~70% of the endogenous TnCs (20).

### *Protein preparation and phosphorylation*

All proteins were cloned from a rat cDNA library (BioChain) into a modified pET6a vector fused to an N-terminal hexa-histidine-tag followed by TEV cleavage site. C1mC2 serine-to-aspartate (S-D) mutations were introduced according to previously published protein primary sequences (8) and introduced by site-directed mutagenesis. Protein constructs were expressed in BL21(DE3)-RIPL cells (Agilent Technologies) followed by affinity chromatography on HisTrapFF columns (GE Healthcare) and removal of the tag-sequence by TEV protease treatment. Proteins were further purified by ion-exchange chromatography (IEC) on MonoS columns (GE Healthcare), concentrated to >100  $\mu$ mol/L, snap frozen in liquid nitrogen and stored in aliquots at -80°C for experiments. Myosin S2 $\Delta$  was cloned from a rat heart cDNA library (BioChain) into a modified pET6a vector, and expressed and purified as described above, except that IEC was instead performed on a MonoQ column. Purity was estimated by SDS-PAGE and ESI mass spectrometry to >95%. C1mC2 was phosphorylated by protein kinase A (PKA) and purified as described previously (5). Briefly, 50  $\mu$ mol/L C1mC2 in PKA assay buffer (composition in mmol/L: 20 Tris-HCl, 50 NaCl, 2 MgCl<sub>2</sub>, 0.1 EDTA, 1 DTT) was incubated with 2000 U/ml of the catalytic subunit of PKA for 30-60 min at 30°C. The reaction was followed by Phostag<sup>TM</sup>-SDS-PAGE and ESI mass spectrometry (Fig. S1). The mainly tris-phosphorylated C1mC2 was further purified by IEC on MonoS, and homogeneity determined by Phostag<sup>TM</sup>-SDS-PAGE and ESI mass spectrometry to be >95%. Prolonged incubation of C1mC2-3P with demembranated trabeculae did not result in any significant protein dephosphorylation (Fig. S1B). For experiments, aliquots of proteins were thawed on ice, and gel-filtered and dialysed against new buffer prior to each experiment.

### *Fluorescence polarization experiments*

Activation protocols and composition of experimental solutions were identical to those described previously for fluorescence polarization experiments (21). Polarized fluorescence intensities were measured as described previously for skeletal and cardiac muscle fibres. Trabeculae were mounted between a strain gauge force transducer (KRONEX, Oakland, California 94602, USA; model A-801,

resonance frequency ~2 kHz) and motor (Aurora Scientific, Dublin, D6WY006, Ireland; Model 312C). Fluorescence emission from BSR-cRLCs and BR-cTnCs in trabeculae was collected by a 0.25 N.A. objective using an excitation light beam in line with the emission path. The polarization of the excitation and emitted beams was set either parallel or perpendicular to the trabecular axis, allowing determination of the order parameter  $\langle P_2 \rangle$  that describes the dipole orientations in the trabeculae (22). The sarcomere length of trabeculae was measured by laser diffraction in relaxing solution prior to each activation. Activating solution contained (in mmol/L): 25 imidazole, 15 Na<sub>2</sub>CrP, 58.7 KPr, 5.65 Na<sub>2</sub>ATP, 6.3 MgCl<sub>2</sub>, 10 CaCl<sub>2</sub>, 10 K<sub>2</sub>EGTA, 1 DTT, pH 7.1. Each activation was preceded by a 2-min incubation in pre-activating solution (composition in mmol/L: 25 imidazole, 15 Na<sub>2</sub>CrP, 108.2 KPr, 5.65 Na<sub>2</sub>ATP, 6.3 MgCl<sub>2</sub>, 0.2 K<sub>2</sub>EGTA, 1 DTT, pH 7.1). Isometric force and steady-state fluorescence polarization values were measured once steady force had been established.

#### ***Native thin filament co-sedimentation***

Native thin filaments (NTF) were prepared according to previously published protocols (23) and co-sedimentation experiments performed as described in (15).

#### ***Microscale thermophoresis***

Microscale thermophoresis experiments were performed on a Monolith NT.115 instrument (NanoTemper, Cambridge CB3 0AX, United Kingdom) in interaction buffer containing 20 mmol/L MOPS pH 7, 1 mmol/L EDTA, 50 mmol/L KCl, 1 mmol/L DTT and 0.05% (v/v) Tween-20. Myosin S2Δ was labelled with Alexa 647-NHS (Molecular Probes Inc, Thermo Fischer Scientific, Paisley, PA4 9RF, United Kingdom) according to manufacturer's instructions, and dye incorporation (efficiency of ~80%) confirmed by HPLC and ESI-mass spectrometry. All proteins were either gel-filtered into and/or extensively dialysed against interaction buffer. Titration experiments were performed with a fixed Alexa 647 – myosin S2Δ concentration of 100 nmol/L in standard treated capillaries. For experiments with isolated m-motif, the pH of the MST buffer was adjusted to 6.2 and experiments were performed in enhanced gradient standard capillaries.

#### ***Statistical analysis***

Statistical significance of difference between groups was assessed with a one-way ANOVA followed by Turkey's post-hoc test. Details of significance levels are shown in the figure captions.

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## **Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

## **Author contributions**

TK designed research; TK and SP performed research; IS and YBS contributed new reagents/analytic tools; TK, SP and MI analysed data; and TK and MI wrote the paper.

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## FOOTNOTES

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The abbreviations used are: BSR, bifunctional sulphorhodamine; C1mC2, fragments of cardiac myosin binding protein-C containing the Ig-domains C1 and C2, and the intervening m-domain; cMyBP-C, cardiac isoform of myosin binding protein-C; cRLC, cardiac isoform of myosin regulatory light chain; cTnC cardiac isoform of troponin C; IT-arm, core domain of troponin containing troponin I and troponin T; PKA, protein kinase A; PKA, Protein Kinase A or cAMP-dependent protein kinase; S-D, serine-to-aspartate

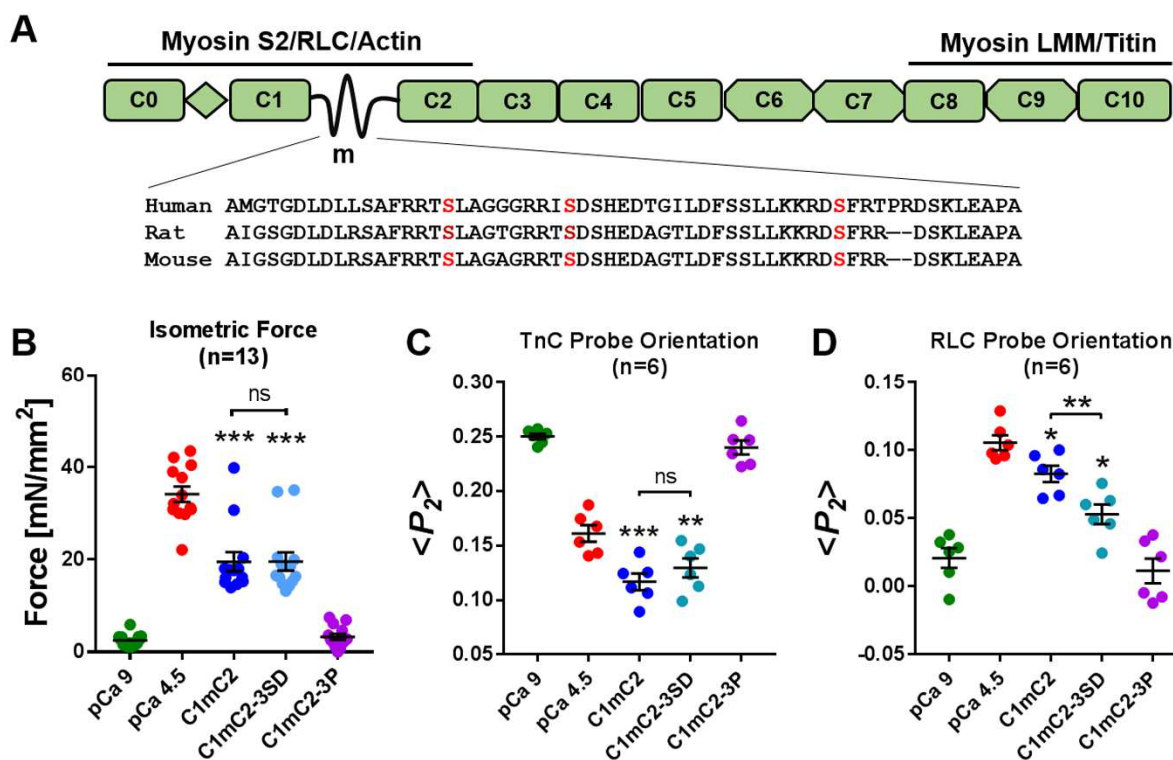
## Tables and Table Legends

**Table 1.** Summary of equilibrium dissociation constants ( $K_d$ ) for different cMyBP-C constructs for myosin S2 $\Delta$  as measured by Microscale Thermophoresis.

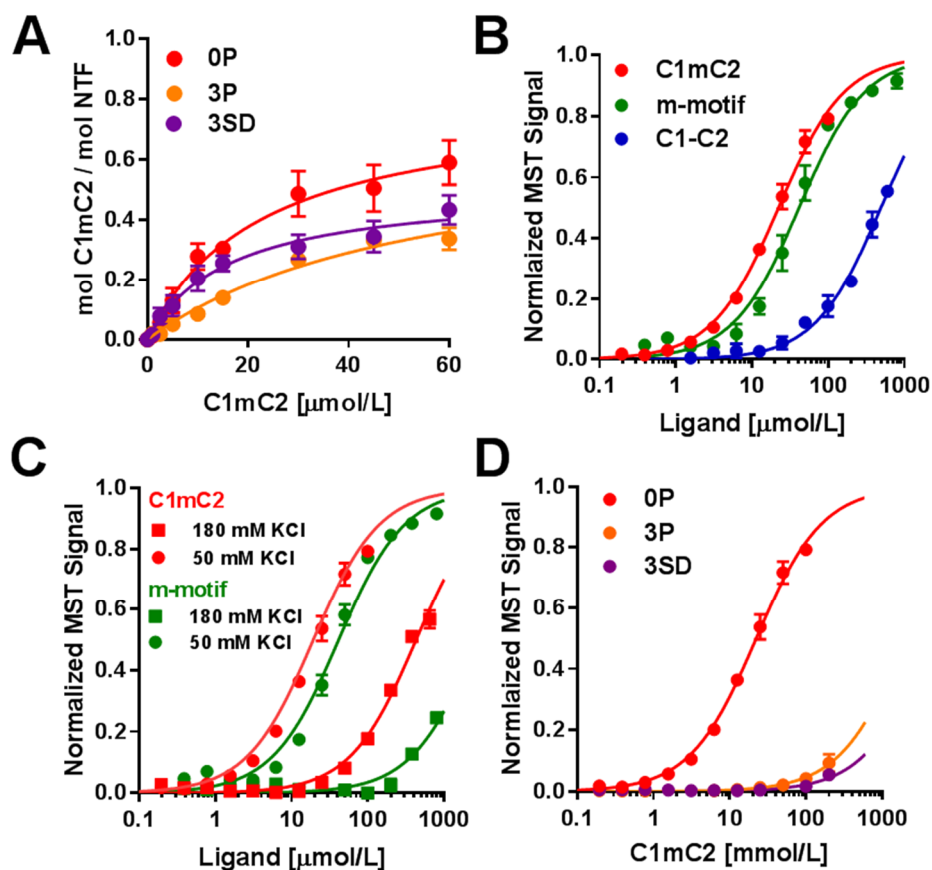
Ligand	T [°C]	KCl [mmol/L]	$K_d$ [ $\mu$ mol/L]
<b>C1mC2</b>	21	50	106.2 $\pm$ 11.2
	25	50	58.5 $\pm$ 8.5
	30	50	21.3 $\pm$ 3.5
	30	180	364.5 $\pm$ 10.6
<b>C1-C2</b>	30	50	477.4 $\pm$ 6.7
<b>m-motif</b>	30	50	41.1 $\pm$ 2.3
	30	180	cd
<b>C1mC2-3P</b>	30	50	cd
<b>C1mC2-3SD</b>	30	50	cd

Means  $\pm$  SEM (n=4-6). cd -  $K_d$  cannot not be reliably determined

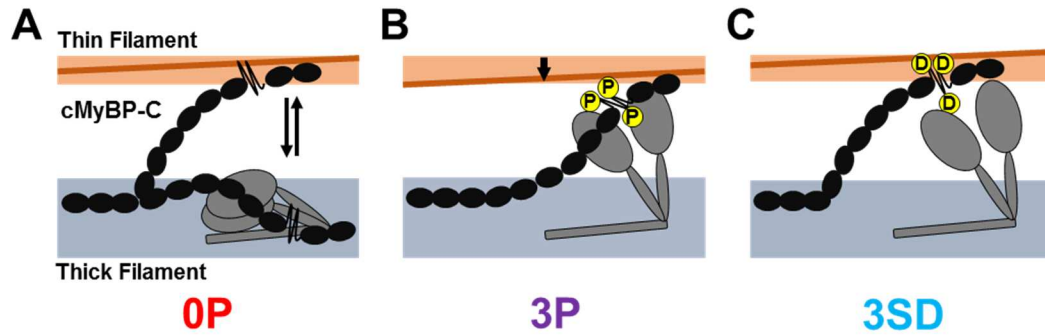
## Figures and Figure Legends



**Figure 1.** Effect of phospho-mimetic substitutions in cMyBP-C on isometric force, and thin and thick filament structure in cardiac muscle. (A) Schematic of cMyBP-C's domain organisation and known protein interactions, with a sequence alignment for the cardiac specific m-motif containing the phosphorylatable serine residues (LMM, light mero-myosin; RLC, regulatory light chain; myosin S2, myosin sub-fragment 2). (B) Isometric force, (C) TnC probe orientation and (D) RLC probe orientation of ventricular trabeculae in relaxing conditions (pCa 9, green), at full calcium activation (pCa 4.5, red), and during incubation in relaxing solution (pCa 9) containing 40  $\mu\text{mol/L}$  C1mC2 (blue), phospho-mimetic C1mC2-3SD (turquoise) or PKA tris-phosphorylated C1mC2-3P (purple). Mean  $\pm$  SEM with the number of trabeculae (n) indicated in each panel. Statistical significance of differences between groups was assessed with a one-way ANOVA followed by Turkey's post-hoc test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns-not significant.



**Figure 2.** Binding of cMyBP-C N-terminal domains to native thin filaments (NTF) and myosin S2 $\Delta$ . (A) NTF co-sedimentation binding data for C1mC2 (red), C1mC2-3P (orange) and C1mC2-3SD (purple). (B) Normalized MST binding curves for C1mC2 (red), isolated m-motif (green) or C1-C2 (blue) titrated against myosin S2 $\Delta$ . (C) Normalized MST curves for C1mC2 (red) and m-motif (green) binding to myosin S2 $\Delta$  in the presence of 50 mM KCl (circles) and 180 mM KCl (squares). (D) Binding of C1mC2 (red), C1mC2-3P (orange) and C1mC2-3SD (purple) to myosin S2 $\Delta$ . For all experiments: Means  $\pm$  SEM, n=4-8.



**Figure 3.** Model for the effect of phosphorylation or serine-aspartate substitutions on cMyBP-C function. (A) In the unphosphorylated state, cMyBP-C binds both thin and thick filaments stabilizing their ON and OFF states, respectively. cMyBP-C inhibits myosin head domains (grey) by stabilizing their folded OFF state and activates the thin filament by moving tropomyosin (brown) away from its blocked position towards the open position. (B) PKA tris-phosphorylation (P) abolishes both the activating and inhibitory effects of cMyBP-C on the thin and thick filament, respectively. (C) In contrast, serine-to-aspartate substitutions (D) abolish the inhibitory interaction between cMyBP-C and myosin, but do not abolish the activating interaction with the thin filament.

**Structural and functional effects of myosin binding protein-C phosphorylation in heart muscle are not mimicked by serine-to-aspartate substitutions**

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